Solid-phase biosynthesis on high performance thin-layer plates of blood group glycosphingolipids II

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A new method for the study of carbohydrate chain biosynthesis of amphipathic glycosphingolipids is presented. The method takes advantage of the thin-layer plate which is used as a solid matrix for precursor glycosphingolipids in biosynthetic experiments after chromatographic development of the precursor sample. The method is shown to be simple, fast and sensitive. It minimizes the risk of adding unwanted exogenous precursors and abolishes the need for tedious purification of products after incubation. The method opens up new possibilities for the biosynthetic study of mixtures of glycosphingolipids.

Glycolipid *Fucolipid* *Blood group* *Thin-layer chromatography* *Glycosyltransferase* *Solid-phase biosynthesis*

1. INTRODUCTION

Glycosyltransferases taking part in glycoprotein biosynthesis have been detected, purified and characterized from a number of sources, but very little work has been published on the biosynthesis in vitro of blood group glycosphingolipids [1,2]. Most biosynthetic experiments with glycosphingolipid precursors have been carried out by studying the transfer of a radioactive sugar unit from a sugar nucleotide donor to a glycolipid acceptor in the presence of, for example, an enzyme (purified or non-purified), detergents or buffer [2]. The limited availability of highly purified and structurally characterized glycosphingolipid acceptors is one major factor determining the number and type of experiments that can be done in this area. Several methodological difficulties in the handling of amphipathic molecules may also be of influence. As thin-layer chromatography has recently [3–6] been shown to be a highly valuable tool in different binding assays (antibodies, lectins, toxins) it became natural to also test the thin-layer plate as a solid matrix for glycosphingolipid precursors in biosynthetic experiments.

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2. MATERIALS AND METHODS

HPTLC plates, precoated with silica gel 60 (Merck) on glass plates were used. After chromatographic development of the sample(s) in an appropriate solvent, the plate was dried and treated sequentially with polyisopropylmethacrylated and 5% bovine serum albumin in phosphate-buffered saline as in [6]. The thin-layer plate was then covered with incubation medium, approx. 1 ml/10 cm^2 (see below), and left at 37°C for 3–4 h in saturated humid atmosphere. After incubation, the thin-layer plate was washed carefully 6 times with phosphate-buffered saline and left to dry in the air. The dry plate was then subjected to autoradiography for 4–8 days using Kodak XAR-5 X-ray film and a suitable developer.

The incubation medium was composed essentially as in [7], and contained in a final volume of 1 ml: 650 μl of enzyme preparation, about 1.3×10^6 cpm of GDP-L-[^{14}C]fucose, 25 μl Tris–HCl buffer

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(pH 7.5), 10 μmol NaN_3, 10 μmol ATP, 7.5 μmol $MgCl_2$, 0.1% Triton X-100.

A microsomal preparation from pig intestinal mucosa was used as enzyme source and was prepared as follows: small intestine from pig was obtained fresh from the local slaughterhouse. The intestine was washed with saline to remove faeces and the mucosa was scrapped off with a spoon and stored at −70°C before use. Approx. 2 ml of mucosa was thawed and washed 3 times with 10 ml of phosphate-buffered saline (centrifugation 5 min in a laboratory bench centrifuge between each washing). The washed mucosa was then homogenized in 15 ml of ice-cold phosphate-buffered saline using a Dounce homogenizer (pestle B, 25 strokes) at 4°C. The homogenate was centrifuged at 450 × *g* for 10 min at 4°C. The pellet was discarded and the supernatant was further centrifugated at 4000 × *g* for 5 min at 4°C. The pellet was again discarded and the supernatant was finally centrifuged at 30 000 × *g* for 15 min at 4°C. The supernatant was discarded and the pellet resuspended in 15 ml of PBS and frozen to −70°C before use. Protein concentration varied between 1–2.5 mg/ml for different preparations.

Total non-acid glycosphingolipid fractions from plasma of individual blood donors were prepared as in [8]. Lactotetraosylceramide was obtained from human meconium [9]. Gangliotetraosylceramide was obtained by acid treatment of GM_1 ganglioside from bovine brain. The major product was purified to homogeneity by DEAE-column chromatography and silicic acid chromatography.

3. RESULTS

Fig. 1 shows the result of a biosynthetic experiment in the solid phase on a thin-layer plate after chromatographic development of the glycosphingolipid precursor fractions. Total non-acid glycosphingolipid fractions from plasma of 4 different blood donors (for blood group ABO, Lewis and secretor typing, see fig.1) were used as precursor mixtures, a microsomal preparation from pig small intestinal mucosa as enzyme source, and GDP-L-[^{14}C]fucose as activated sugar. The anisaldehyde-visualized section of the thin-layer plate shows the precursor fractions after chromatographic development and the figures to the left indicate the approximate number of carbohydrate

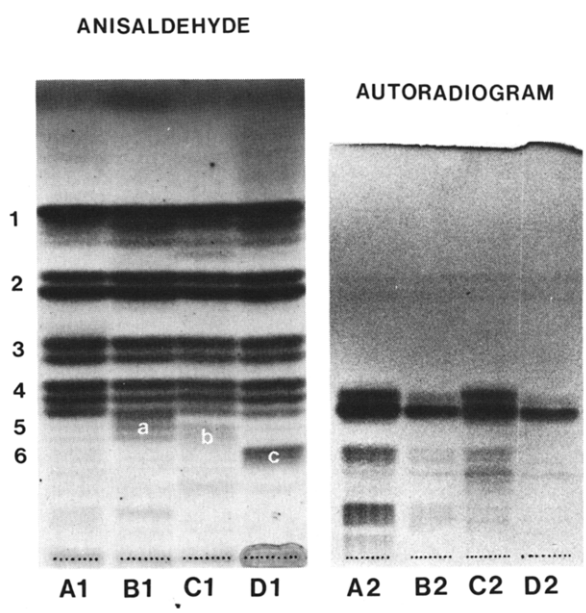

Fig. 1. Total non-acid glycosphingolipid fractions of plasma of 4 different blood donors (for blood group ABO, Lewis and secretor typing, see below photograph) were applied twice on a thin-layer plate: approx. 30 μg of each (A1,B1,C1 and D1) and approx. 10 μg of each (A2,B2,C2 and D2). After chromatographic development in chloroform:methanol:water (60:35:8, by vol.) one section of the thin-layer plate (A1,B1,C1 and D1) was visualized with the anisaldehyde reagent [19]. The figures on the left hand side indicate the approximate number of sugars in the glycolipid carbohydrate chain. a, b, and c indicate tentatively the thin-layer position of the blood group H, type 1 chain pentaglycosylceramide, the blood group Le^a pentaglycosylceramide and the blood group Le^b hexaglycosylceramide [8,10–12], respectively. The remaining section (lanes A2,B2,C2 and D2) was incubated with an enzyme preparation from pig intestinal mucosa in the presence of GDP-L-[^{14}C]-fucose for 3 h and subsequently subjected to autoradiography for 8 days (see section 2).

units in the glycosphingolipid carbohydrate chain. The location of the blood group Le^b hexaglycosylceramide, the blood group Le^a pentaglycosylcer-

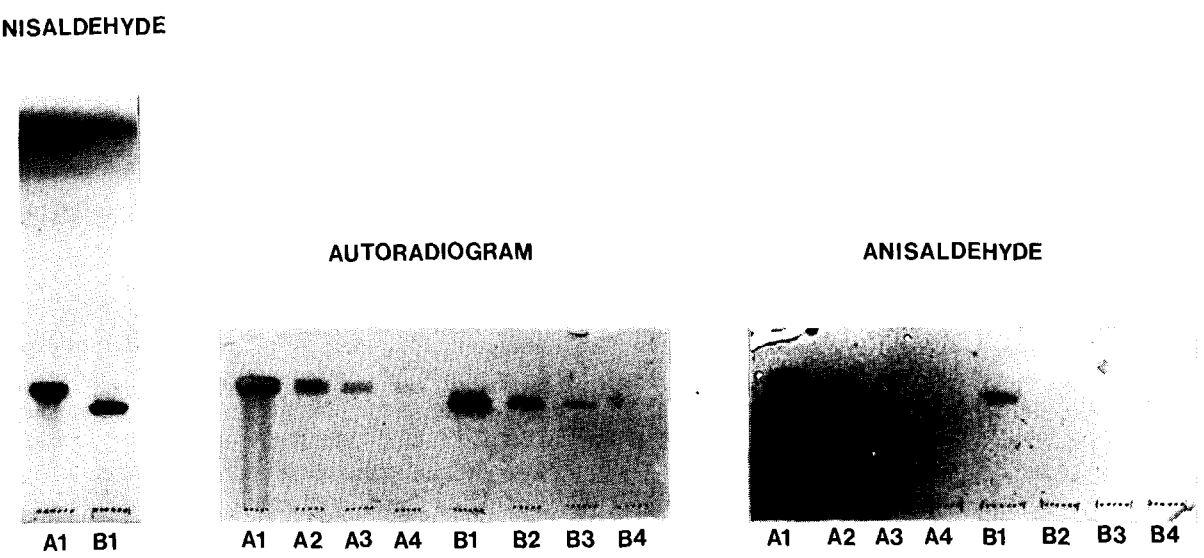

Fig 2. The following glycosphingolipid precursors were applied on a thin-layer plate: Lactotetraosylceramide: A1, 2 μg; A2, 0.2 μg; A3, 0.02 μg; A4, 0.002 μg. Gangliotetraosylceramide: B1, 2 μg; B2, 0.2 μg; B3, 0.02 μg; B4, 0.002 μg. After chromatographic development in chloroform:methanol:water (60:35:8, by vol.) one section of the thin-layer plate (A1 and B1) was visualized by using the anisaldehyde reagent [19]. The remaining section A1-A4, B1-B4 was incubated with an enzyme preparation from pig intestinal mucosa in the presence of GDP-L-[^{14}C]fucose and subsequently subjected to autoradiography for 8 days. To visualize the precursor fractions after autoradiography, the thin-layer plate was extracted several times with diethylether and finally treated with the anisaldehyde reagent.

amide and the blood group H (type 1 carbohydrate chain) pentaglycosylceramide [8,10–12] are also indicated. The autoradiogram, fig.1, shows the biosynthesized, fucosylated, products with R_f values coinciding with their corresponding precursors. The bands seen on the autoradiogram are as clear and distinct as the ones shown after anisaldehyde visualization and both quantitative and qualitative differences are seen between the different individual plasma fractions. No bands are seen on the autoradiogram over the cerebroside region (1 sugar) or the globotriaosylceramide region (3 sugars). Weaks bands are, however, seen over the lactosylceramide region (2 sugars) indicating fucosylation of this structure.

Fig. 2 shows a similar experiment using dilution series of highly purified glycosphingolipid precursors, lactotetraosylceramide and gangliotetraosylceramide. The autoradiogram shows that it is possible to detect a fucosylated product using as little as 2 ng of precursor.

4. DISCUSSION

One important aspect to consider when presenting a new non-conventional biosynthetic method is if the specificity of the enzymatic reaction is retained. Ideally a well characterized enzyme preparation of high purity and pure precursors should be used. This is not possible at the present stage. However, there is considerable indirect evidence to indicate that the specificity is retained. The enzyme preparation from pig intestinal mucosa is crude but the use of GDP-L-[^{14}C]fucose restricts the study to fucosyltransferases. It is well known that porcine submaxillary gland contains large amounts of a β-galactoside $\alpha 1 \rightarrow 2$ fucosyltransferase [13] and that porcine intestinal mucosa contain relatively large amounts of a blood group H pentaglycosylceramide with a type 1 chain ([14], unpublished). A Fuc$\alpha 1 \rightarrow 2$ transferase is thus expected in the intestinal mucosa of pig. Using the experimental conditions described and some 15

different highly purified well characterized glycosphingolipid precursors, the acceptor specificity of the enzyme preparation described, on a semiquantitative basis, is very similar to that described [15] for the Fucα1→2 transferase of porcine submaxillary gland (to be published).

Fucosylation (Fucα1→2) of both lactotetraosylceramide and gangliotetraosylceramide is thus expected [15] (fig. 2). The sensitivity, 2 ng, appears quite impressive but is of course dependent on the enzyme preparation. Using fresh saliva as the source of fucosyltransferase a similar detection level is about 0.1 μg (to be published).

Fig. 1 illustrates the possibility of performing biosynthetic experiments using mixtures of acceptor glycosphingolipids and yet on the thin-layer plate permitting separation and possibly identification of the precursors. This facility can be very useful in biosynthetic experiments in determining if the major component or a minor impurity in a precursor fraction is the true glycolipid acceptor. Furthermore, as in fig. 1, the total number of 'theoretical' precursor glycosphingolipids from any organ or individual could be exposed for a given enzyme preparation in the presence of a suitable ^{14}C-labelled sugar nucleotide. The results, although complex, are rather informative as the appearance of a band on the autoradiogram is interrelated to both the separation of possible precursors and the thin-layer mobility of the special precursor in question. If the structure of the precursor is known, the identification of the product is within reach. If the precursor is not known it may be profitable to try to isolate the presumably new glycolipid formed. This experimental model may be advantageous as a screening procedure when working with problems concerned with the understanding of how blood group-specific glycosphingolipid patterns come about and how genetic differences are expressed on the glycosphingolipid level. Fig. 1 thus tentatively shows that Fucα1→2 transferase acceptors are present in the plasma of all 4 individuals but differ both quantitatively and qualitatively possibly due to the interaction of Lewis and secretor genes and perhaps also due to other unknown genes. The presence of very complex thin-layer chromatography (slow-moving) Fucα1→2 precursor glycosphingolipids in the Le(a-b-) non-secretor individual has not been shown before. The autoradiogram in fig. 1 also shows very faint bands for all 4 individuals over the lactosylceramide region. This most probably indicates the biosynthesis of a II^2-α-fucosyllactosylceramide. This structure has been described in rat intestinal epithelial cells [16] and in mouse B cell hybridomas [17].

The strategy behind most glycosphingolipid experiments is to study the transfer of a radioactively labelled sugar nucleotide to a glycosphingolipid acceptor [1,2]. To really ascertain that the biosynthesized product is a glycosphingolipid in conventional non-solid phase experiments a cumbersome and tedious extraction and preparation procedure is needed [18]. Furthermore when using crude enzyme preparations there is a risk of adding unwanted exogenous precursor glycolipids. The described method minimizes this risk and abolishes the need for glycosphingolipid preparation after incubation.

The advantages of the method combined with its relative swiftness and technical simplicity should make it at least complementary to other methods in the biosynthetic field.

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